

N° d'Enregistrement
au C.N.R.S. : A.O. 4994

ARTICLE PRINCIPAL

Recouvrant en partie

LA THESE DE DOCTORAT D'ETAT ES SCIENCES NATURELLES

présentée

A LA FACULTE DES SCIENCES DE PARIS

par

Pierre ROUGET

pour obtenir le grade de Docteur Es-Sciences

Sujet de la thèse : PROPRIETES ET MECANISME D'ACTION DE LA
LEUCYL-tARN SYNTHETASE DE E. COLI

Soutenue le 6 Novembre 1970 devant la Commission d'Examen :

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Leucyl-tRNA Synthetase

Purification of Two Interconvertible Forms and Evidence for an Interconversion Factor

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(Received February 19/April 3, 1970)

Two forms of leucyl-tRNA synthetase of *Escherichia coli* B were separated and purified about 850-fold to apparent homogeneity. Their molecular weights were identical, 104 000, as determined by Sephadex filtration and polyacrylamide electrophoresis.

One of these forms catalyzed the leucine-dependent ATP-PP_i exchange reaction but was unable to transfer leucine to tRNA even though it could form a complex with tRNA^{Leu}. The other form was active for both ATP-PP_i exchange and charge of tRNA.

Evidence was obtained for the interconversion between these two forms of leucyl-tRNA synthetase and for the existence in the *E. coli* 105 000×*g* supernatant of a factor stimulating this interconversion; during the incubation of one of these forms with the supernatant, the other form appeared and an apparent equilibrium between the two forms was observed.

Berg *et al.* [1] and, more recently, Bennett [2] have partially purified the *E. coli* leucyl-tRNA synthetase. Yamane and Sueoka [3,4] and Bennett [2] have shown that different leucine-specific tRNAs were acylated by a single enzyme, whereas on the basis of results obtained with fractions eluted from chromatographic columns by step-wise procedures, Yu [5] and Strehler *et al.* [6] have concluded to the existence of several leucyl-tRNA synthetases, using *E. coli* and rabbit tissues respectively.

In the present work, evidence for the existence of two interconvertible forms of leucyl-tRNA synthetase was obtained. Each form was purified to homogeneity with respect to acrylamide gel electrophoresis. One of these forms catalyzed the leucyl-dependent ATP-PP_i exchange reaction but was unable to transfer the amino acid to tRNA whereas the other form was able to catalyze both reactions. However both forms could give rise to complexes with tRNA_I^{Leu}, as well as with tRNA_{II}^{Leu}.

Evidence was obtained for the presence, in the *E. coli* 105 000×*g* supernatant, of a factor which stimulates the interconversion between the two forms of leucyl-tRNA synthetase.

Unusual Abbreviations. tRNA^{Leu}, leucine acceptor tRNA; tRNA_I^{Leu}, peak I fraction of leucine specific tRNA as described in [8]; tRNA_{II}^{Leu}, peak II fraction of leucine specific tRNA.

Enzymes. Leucyl-tRNA synthetase of L-leucine: tRNA ligase (AMP): (EC 6.1.1.4); tyrosyl-tRNA synthetase or L-tyrosine: tRNA ligase (AMP) (EC 6.1.1.1); horseradish peroxidase (EC 1.11.1.7); *E. coli* alkaline phosphatase (EC 3.1.3.1); yeast alcohol dehydrogenase (EC 1.1.1.10); pepsin (EC 3.4.4.1); hexokinase (EC 2.7.1.1).

MATERIAL AND METHODS

MATERIAL

E. coli B cells were grown on a yeast extract-glucose-potassium phosphate medium, harvested in late log phase, centrifuged and stored at -20°.

Crude tRNA from *E. coli* B was purchased from General Biochemicals or prepared according to the method of Nathans and Lipmann [7], tRNA_I^{Leu} and tRNA_{II}^{Leu} (about 40% pure, Goldstein *et al.* [8]) were a gift of Dr. M. Yaniv [9].

[¹⁴C]Leucine 120 mC/mmol and [³²P]pyrophosphate were obtained from the Department de Biologie du Commissariat à l'Energie Atomique (Saclay).

[³H]ATP 4 C/mmol was from Schwartz-Bio-research and diluted to 250 mC/mmol.

ATP was purchased from Pabst Laboratories; sodium pyrophosphate from Hopkin and Williams and 2-mercaptoethanol was a product of Fluka.

Unlabeled leucine was obtained from Mann Research Laboratories and rechromatographed twice on Whatman paper 3 in *n*-butanol saturated with 2 N NH₄OH and in pyridine-acetic-acid-H₂O (50:35:15).

DEAE-cellulose was purchased from Bio-Rad Laboratories and hydroxylapatite from Clarkson Chemical Corporation. Acrylamide and bis-acrylamide were purchased from Fluka and recrystallized in chloroform and acetone respectively.

Horseradish peroxidase, *E. coli* alkaline phosphatase, yeast alcohol dehydrogenase and pepsin were obtained from Worthington Biochemical Corporation.

Cytochrome *c* and hexokinase were from Boehringer Soehne (Ingelheim); bovine serum albumin was from Sigma.

Purified tyrosyl-tRNA synthetase was a gift of S. Chousterman.

Protamine sulfate was from Mann Research Laboratories.

METHODS

Enzyme Assay

ATP-PP_i Exchange Reaction. The ATP-pyrophosphate exchange was determined according to a slight modification of the procedure described by Waller *et al.* [10]. The incubation mixture contained in a total volume of 1 ml: 100 μ moles Tris-Cl pH 7.4, 5 μ moles MgCl₂, 2 μ moles ATP, 2 μ moles [³²P]pyrophosphate, 2 μ moles leucine, 5 μ moles 2-mercaptoethanol and limiting amounts of enzyme. The mixture was incubated at 37° and aliquots were removed at various times. The reaction was stopped by the addition, successively, of 0.7 ml 5% perchloric acid, 0.3 ml of an aqueous suspension of Norite (100 mg/ml) and 1.5 ml 0.1 M sodium pyrophosphate. After thorough mixing and centrifugation, the supernatant was removed and the Norite was washed twice with 2 ml of 0.1 M sodium pyrophosphate. ATP was eluted with 2 ml of 0.3 M ammonium hydroxide in 50% ethanol. After centrifugation, 1 ml of the supernatant was dried and counted in a Nuclear Chicago gas-flow counter.

One exchange unit of enzyme is defined as causing the incorporation of 1 μ mole of [³²P]PP_i into ATP in 15 min at 37° under the described conditions.

Protein was determined by the method of Lowry *et al.* [11].

Leucyl-tRNA Formation. The incubation mixture contained in a total volume of 1 ml: 100 μ moles Tris-Cl pH 7.4, 5 μ moles MgCl₂, 2 μ moles ATP, 5 μ moles 2-mercaptoethanol, 1 mg crude tRNA (or 0.1 mg purified tRNA), 10 nmoles [¹⁴C]leucine and limiting amounts of enzyme. The mixture was incubated at 37° and aliquots were withdrawn at various times; the reaction was stopped by the addition of 1 mg of yeast RNA as carrier and 2 ml of 0.5 M perchloric acid containing 2 mg/ml of unlabeled leucine. The precipitate was washed twice with 0.5 M perchloric acid containing 2 mg/ml of leucine and once with cold ethanol-ether (1:1, v/v). The precipitate was then dissolved in formic acid and, after evaporation on a planchet, counted as indicated above.

One charge unit of enzyme is defined as the amount of enzyme catalysing the formation of 10 nmoles of leucyl-tRNA in 15 min at 37°.

Preparation of Leucyl-tRNA

Leucyl-tRNA was prepared according to Chapeville *et al.* [12]. The relative proportion of the reagents were the same as described above, except for tRNA: when purified tRNA was used, its concentration was the same as that of the analytical assay whereas with crude tRNA the concentration was five-fold higher.

After 30 min of incubation at 37°, pH was adjusted to 5 by the addition of 0.1 volume of 20% potassium acetate containing 2 mg/ml of unlabeled leucine and one volume of 90% phenol was added. After thorough mixing and centrifugation, the aqueous phase was collected and the phenolic phase was washed twice with water. The aqueous phases were combined, 0.1 volume of 20% potassium acetate and two volumes of cold ethanol were added. After 2 hours at -20°, the leucyl-tRNA was centrifuged, dissolved in 0.1 mM potassium acetate, dialyzed against the same solution and stored at -20°.

Formation of Complexes between the Enzyme and ATP or Leucyladenylate

The formation of these complexes was performed as already described [13]. The incubation mixture contained in a total volume of 1 ml: 50 μ moles Tris-Cl pH 7.4, 10 μ moles MgCl₂, 5 μ moles 2-mercaptoethanol, 100 nmoles [³H]ATP, 0.5 mg enzyme, 100 nmoles [¹⁴C]leucine were added only for the formation of the enzyme-leucyladenylate complex. After incubation for 10 min at 20°, the mixture was chilled to 0° and loaded onto a Sephadex G-75 column (1×40 cm) previously equilibrated with 0.01 M Tris-buffer pH 7.4 and 5 mM 2-mercaptoethanol. Fractions of 1 ml were collected and aliquots removed for counting in a solution of 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazolyl-2)-benzene in toluene-ethanol.

Formation of Complexes between the Enzyme and Leucyl-tRNA

The incubation mixture (0.4 ml) contained: 20 μ moles sodium cacodylate buffer pH 6, 4 μ moles MgCl₂, 4 μ moles 2-mercaptoethanol, 10 μ g leucyl-tRNA synthetase and [¹⁴C]leucyl-tRNA in the presence or absence of tRNA as indicated in tables. After 5 min of incubation at 4°, the reaction mixture was passed through a nitrocellulose filter (Schleicher & Schüll B₆) using a method similar to that described by Yarus and Berg [14] for the isoleucyl-RNA synthetase. The filters were washed with 20 ml of 0.05 M cacodylate buffer pH 6 containing 10 mM MgCl₂ and 10 mM 2-mercaptoethanol; they were dried and counted in the presence of a solution of 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazolyl-2)-benzene in toluene.

Sucrose Gradient Centrifugations

Sucrose gradient centrifugations were carried out according to Martin and Ames [15] with gradients of 4.8 ml from 5–20% sucrose in 0.02 M potassium phosphate pH 6.5, 0.1 M KCl and 0.01 M 2-mercaptoethanol. Samples of purified enzyme, previously dialyzed against the same buffer were loaded on the gradients. After centrifugation at 39000 rev./min, about 40 fractions were collected and assayed for protein content and enzyme activity.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis were performed according to Jovin *et al.* [16] using the Tris-glycine buffer system.

Maintaining a constant ratio of bisacrylamide to acrylamide (0.8:30), various concentrations of acrylamide were used in order to determine the molecular weights according to the method described by Hedrick and Smith [17].

Sephadex G-200 Gel Filtration

The purified enzyme was dialyzed against a solution containing 0.02 M potassium phosphate buffer pH 6.8, 0.1 M KCl and 0.01 M 2-mercaptoethanol and 0.5 mg of enzyme was applied onto a column of Sephadex G-200 (1.8 × 100 cm) previously equilibrated with the same solution. Fractions of 1 ml were collected at a flow-rate of 7 ml per hour.

The column was calibrated with proteins of known molecular weights previously dialyzed against the same solution.

Cytochrome *c* and rabbit haemoglobin were localized by absorption at 415 nm. *E. coli* alkaline phosphatase was measured according to Garen and Levinthal [18]. Yeast alcohol dehydrogenase was assayed according to Racker [19] and horseradish peroxidase as described by Worthington Biochemical Corporation [20].

Tyrosyl-tRNA synthetase was localized by ATP-PP_i exchange reaction.

RESULTS

PURIFICATION OF LEUCYL-tRNA SYNTHETASE

All steps were performed in the presence of 10 mM 2-mercaptoethanol at 0–4° unless otherwise stated.

Crude Extract

Frozen *E. coli* cell paste (500 g wet weight) was broken and suspended in 250 ml of 0.025 M K₂HPO₄ and the suspension was homogenized at low speed for a few seconds in a Sorvall Omni-Mixer. After the addition of 1 kg of glass beads and of 250 ml of 0.025 M K₂HPO₄, the cells were disrupted at 90 V

for 30 min; 500 ml of 0.025 M K₂HPO₄ were added and the disruption was continued at the same speed for 30 min. The supernatant was decanted the beads were washed twice with 250 ml of 0.025 M K₂HPO₄, and the supernatants were combined and centrifuged at 30000 × *g* for 1 hour.

Autolysis

The crude extract was diluted to 12.5 mg/ml (2160 ml) with 0.025 M K₂HPO₄ and 28 g of K₂HPO₄ were added so as to adjust its concentration to 0.1 M, thereby facilitating the dissociation of the ribosomes and the autodigestion of nucleic acids. The mixture was incubated for 3 hours at 37° at which time the absorbance at 260 nm of the acid soluble fraction reaches 75% of the total absorbance. The mixture was cooled to 4° and the precipitate discarded by centrifugation for 15 min at 15000 × *g*. The total enzyme activity was not strongly affected by this treatment.

Protamine Sulfate Precipitation

Nucleic acids were further eliminated by the slow addition of 220 ml of a 5% protamine sulfate solution pH 7. After stirring during 2 hours, the precipitate was discarded.

Ammonium Sulfate Fractionation

The resulting supernatant (2000 ml) was treated with 352 g of solid ammonium sulfate. After stirring for 30 min, the mixture was centrifuged for 30 min at 15000 × *g*. The precipitate was discarded and 134 g of solid ammonium sulfate were added to the supernatant (40% saturation). The suspension was stirred and the precipitate again removed as above. The supernatant was brought to 50% saturation by the addition of 140 g of ammonium sulfate. After stirring for 30 min, the precipitate was collected and the supernatant was discarded. The precipitate was dissolved in 300 ml of 0.025 M potassium phosphate pH 6.8 and dialyzed three times for 6 hours against 10 liters of the same buffers. The small amount of precipitate which appeared during the dialysis was removed by centrifugation.

DEAE-Cellulose Chromatography

The preceding fraction was loaded onto a DEAE-cellulose column (3 × 40 cm) previously equilibrated with 0.025 M potassium phosphate pH 6.8. The column was washed with 1 l of 0.05 M phosphate buffer pH 6.8 and a linear gradient was applied: the mixing chamber contained 1 l of 0.05 M potassium phosphate pH 6.8 and the reservoir 1 l of 0.3 M of the same buffer. The flow rate was 80 ml/h and 15 ml fractions were collected. Fig. 1 shows that two peaks

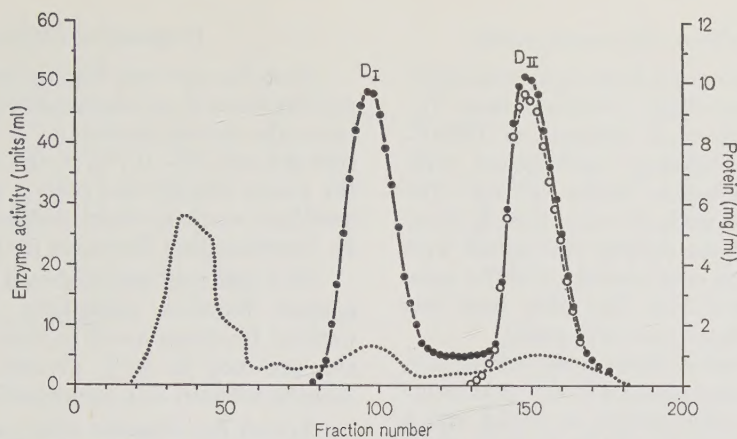


Fig. 1. Chromatography of leucyl-tRNA synthetase on DEAE-cellulose. The ammonium sulfate extract (2800 mg of protein) was applied onto a 3×40 cm column and the enzyme was eluted as described in the text. ●—●, ATP-PP_i exchange; ○---○, leucyl-tRNA formation; ·····, protein

of enzyme appeared with regard to the leucine-dependent ATP-PP_i exchange activity (peaks D_I and D_{II}), only one of which being able to promote the leucyl-tRNA formation (peak D_{II}).

Pools of the active fractions were precipitated separately by the addition of solid ammonium sulfate to 75% saturation. After stirring for 1 hour, each precipitate was collected, dissolved in 100 ml of 0.05 M potassium phosphate pH 6.8 and dialyzed extensively against the same buffer. After this treatment it was noted that the fraction resulting from peak D_I had recovered some activity with regard to the leucyl-tRNA formation: the ratio between charge and exchange activities was about 20% as compared to the ratio observed for D_{II}. On the other hand, in the fraction resulting from peak D_{II} the ratio between charge and exchange activities has decreased by about 25%.

Hydroxyapatite Chromatography

Fractions D_I and D_{II} were applied onto two identical columns of hydroxylapatite (3×40 cm) previously equilibrated with 0.05 M potassium phosphate buffer pH 6.8. The column was then washed with 60 ml of 0.08 M potassium phosphate buffer pH 6.8 and the enzyme was eluted with a linear gradient (1 l in each chamber) of the same buffer, from 0.08 M to 0.25 M. The flow rate was 50 ml/h and 15 ml fractions were collected. Fig. 2 shows that each fraction (D_I and D_{II}) gave two peaks of activity (HyA and HyB) as regards the ATP-PP_i exchange activity and only one peak (HyA) as regards the leucyl-tRNA formation.

It thus appears that fractions D_I and D_{II} resulting from DEAE-cellulose chromatography are to some extent interconvertible.

The active fractions of each peak were pooled separately and precipitated by addition of solid ammonium sulfate to 75% saturation. After stirring

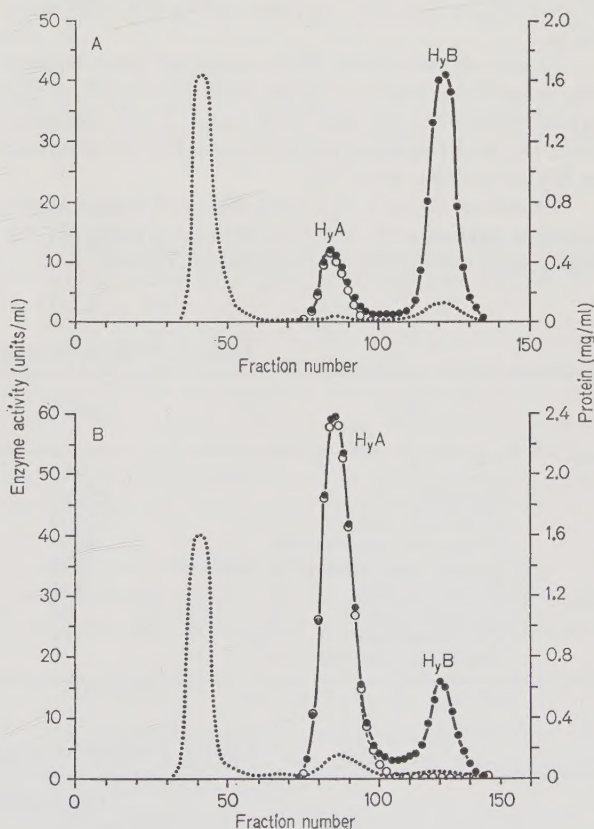


Fig. 2. Chromatography of D_I and D_{II} on hydroxyapatite. D_I (314 mg) or D_{II} (293 mg) were loaded onto a 3×40 cm column and eluted as described in the text. (A) Chromatography of D_I; (B) chromatography of D_{II}. ●—●, ATP-PP_i exchange; ○---○, leucyl-tRNA formation; ·····, protein

for 1 hour, each precipitate was collected, dissolved in 50 ml of 0.025 M phosphate buffer pH 6.8 and dialyzed extensively against the same buffer.

Second DEAE-Cellulose Chromatography

Fraction HyA, obtained by hydroxyapatite chromatography of D_{II}, and HyB, obtained from D_I, were applied to two identical columns of DEAE-cellulose (2×30 cm) previously equilibrated with 0.025 M potassium phosphate buffer pH 6.8. The column was then washed with 300 ml of 0.05 M phosphate buffer pH 6.8 and the enzyme was eluted with linear gradient (350 ml in each chamber) of the same buffer from 0.05 M to 0.3 M. The flow rate was 80 ml/h and 10 ml fractions were collected.

Fraction HyA yielded a minor peak (about 2% of the major peak with regards to ATP-PP_i exchange activity) unable to transfer leucine to tRNA and a major peak capable of promoting both reactions (E_{II}).

Fraction HyB was also resolved in two peaks: the major peak (E_I) was capable of stimulating the ATP-PP_i exchange reaction whereas the minor peak (about 2% of the first one) was active for both reactions.

It was observed that HyA obtained from D_I had the same behaviour on DEAE-cellulose as HyA obtained from D_{II}; on the other hand, HyB resulting from D_{II} had the same behaviour on DEAE-cellulose as HyB resulting from D_I.

Fractions E_I and E_{II} were dialyzed under slight pressure against a 50 mM Tris-370 mM glycine pH 8.3 buffer containing 10 mM 2-mercaptoethanol.

Preparative Electrophoresis

Each fraction was further purified by preparative polyacrylamide gel electrophoresis, using acrylamide-bisacrylamide respectively 7.5–0.2% in the separation gel and 2.5–0.7% in the concentration gel and the above Tris-glycine pH 8.3 buffer. The apparatus used here was somewhat modified from that described by Duesberg and Rueckert [21].

Each fraction was dialyzed under slight pressure against 0.025 M phosphate buffer pH 6.8. The purified fractions could be kept at 0° or at –20° in the presence of 50% glycerol for more than six months without any appreciable loss of activity.

E_I and E_{II} obtained after preparative gel electrophoresis were used in all subsequent experiments, unless otherwise stated.

The purification procedure is summarized in Table 1 for E_I and in Table 2 for E_{II}.

CRITERIA OF PURITY AND MOLECULAR WEIGHT
DETERMINATION

Chromatography on Hydroxyapatite

Re-chromatography on hydroxyapatite on each fraction (E_I and E_{II}) results in a single protein peak of constant specific activity.

Table 1. *Purification steps of leucyl-tRNA synthetase, E_I*

Steps	Protein	ATP-PP _i exchange		
		Total activity	Specific activity	Yield
	mg	units	units/mg	%
Crude extract	27400	32900	1.2	100
Autolysed extract	12000	32400	2.7	98
Protamine supernatant	9050	31700	3.5	96
Ammonium sulfate, 40–50%	2800	27200	9.4	83
DEAE-cellulose	314	11900	38	36
Hydroxyapatite	24	8350	340	25
DEAE-cellulose	12	7250	611	22
Gel electrophoresis	7	6800	970	20

Table 2. *Purification steps of leucyl-tRNA synthetase, E_{II}*

Steps	Protein	ATP-PP _i exchange			Leucyl-tRNA formation			Ratio
		Total activity	Specific activity	Yield	Total activity	Specific activity	Yield	
	mg	units	units/mg	%	units	units/mg	%	Exchange activity Charge activity
Crude extract	27400	32900	1.2	100	175	0.64	100	1.88
Autolysed extract	12000	32400	2.7	98	168	1.4	96	1.93
Protamine supernatant	9050	31700	3.5	96	163	1.8	93	1.94
Ammonium sulfate 40–50%	2800	27200	9.4	83	140	5	80	1.88
DEAE-cellulose	293	13600	47	41	126	43	72	1.09
Hydroxyapatite	28	10100	364	30	101	360	58	1.01
DEAE-cellulose	13	8550	655	26	79	610	45	1.07
Gel electrophoresis	8	8150	1020	25	77	980	44	1.04

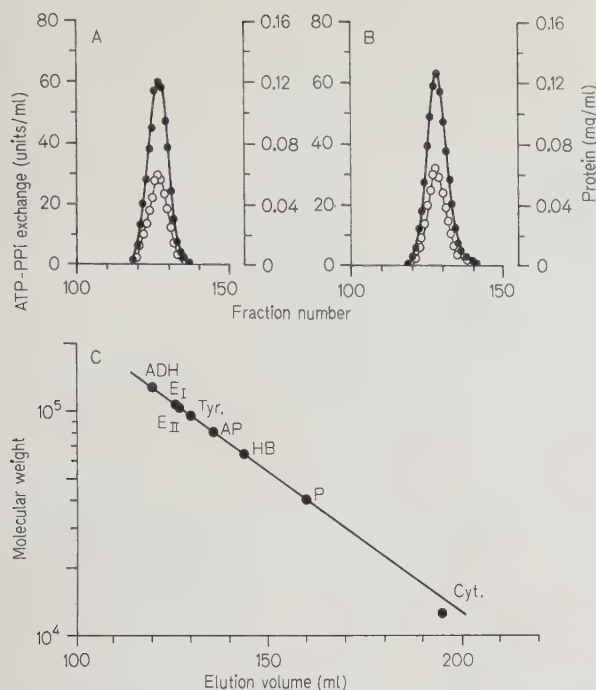


Fig. 3. Filtration on Sephadex G-200. Filtration of E_I (A) and of E_{II} (B): in each case, 0.5 mg of the purified enzyme was applied onto a 1.8 × 100 cm column as described in Methods. The column was previously calibrated with proteins of known molecular weights: yeast alcohol dehydrogenase (ADH), *E. coli* tyrosyl-tRNA synthetase (Tyr), *E. coli* alkaline phosphatase (AP), rabbit hemoglobin (HB), horseradish peroxidase (P) and cytochrome *c* (Cyt). The molecular weights of E_I and E_{II} were determined according to the method of Andrews [22] (C). ●—●, ATP-PP_i exchange; ○—○, protein

Gel Filtration on Sephadex G-200

Each purified fraction, chromatographed on Sephadex G-200 gave a single symmetrical protein peak of constant specific activity as shown in Fig. 3. The estimations of the molecular weights according to Andrews [22] were 106 000 and 104 000 for E_I and E_{II}, respectively.

Sucrose Gradient Centrifugation

Sucrose gradient centrifugation for E_I or E_{II} results in a single symmetrical peak with constant specific activity. The *s*_{20,w} relative to yeast alcohol dehydrogenase, 7.4 S [23], was 5.3 S for E_I as also for E_{II}.

Analytical Gel Electrophoresis

When submitted to disc electrophoresis each purified fraction gave a single band at all the gel concentrations tested, even when as much as 50 μg of protein were loaded onto the gel column (Fig. 5).

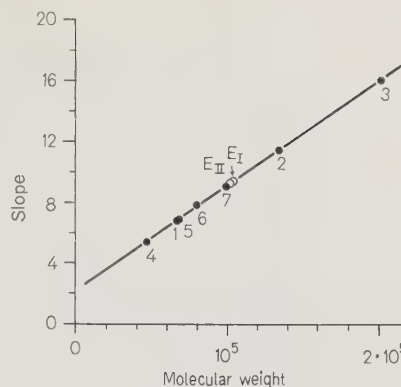


Fig. 4. Molecular weight determination by gel electrophoresis. Proteins were dialyzed against 50 mM Tris, 370 mM glycine pH 8.3 buffer; 50 μg of each protein were loaded on top of the gel columns and electrophorized as described by Hedrick and Smith [17]. Seven gel concentrations were used (4.5, 5.25, 6, 6.75, 7.5, 8.25, 9% acrylamide); the ratios between the migration of protein and the migration of bromophenol blue, *R_m*, were determined; log *R_m* was plotted against gel concentration; straight lines were obtained and their negative slopes were plotted here against molecular weight. 1, 2, 3: Bovine serum albumin monomer, dimer and trimer, respectively; 4: ovalbumine; 5: pepsin dimer; 6: *E. coli* alkaline phosphatase; 7: hexokinase

The molecular weights, estimated according to the method of Hedrick [17] were 104 000 and 102 000 for E_I and E_{II}, respectively, as shown by Fig. 4.

Fig. 5 (A B C D) shows the migration of E_I and Fig. 5 (E F G H) that of E_{II} for different gel concentrations. It can be seen that the migration of E_I was slightly slower than that of E_{II}, indicating a somewhat lower negative charge for E_I than for E_{II}. Fig. 6 shows the separation of E_I and E_{II} from a mixture of both enzymes in the 7.5% acrylamide gel.

EVIDENCE FOR THE EXISTENCE OF TWO INTER-CONVERTIBLE FORMS OF LEUCYL tRNA SYNTHETASE AND FOR THE EXISTENCE OF AN INTERCONVERSION FACTOR

Evidence for Two Forms of the Enzyme

The chromatographic steps of purification of leucyl-tRNA synthetase showed that the enzyme was resolved in two peaks.

When submitted to DEAE-cellulose chromatography (Fig. 1) the leucine-dependent ATP-PP_i exchange activity was resolved into two peaks of about the same intensity (fractions D_I and D_{II}); only one of which (D_{II}) was also able to transfer leucine to tRNA.

After ammonium sulfate precipitation or storing for one week at 4°, D_I yielded two peaks on hydroxyapatite: the first (HyA) being able to charge tRNA, the second (HyB) catalysing only the ATP-PP_i exchange reaction. Here HyA was about 20% of HyB

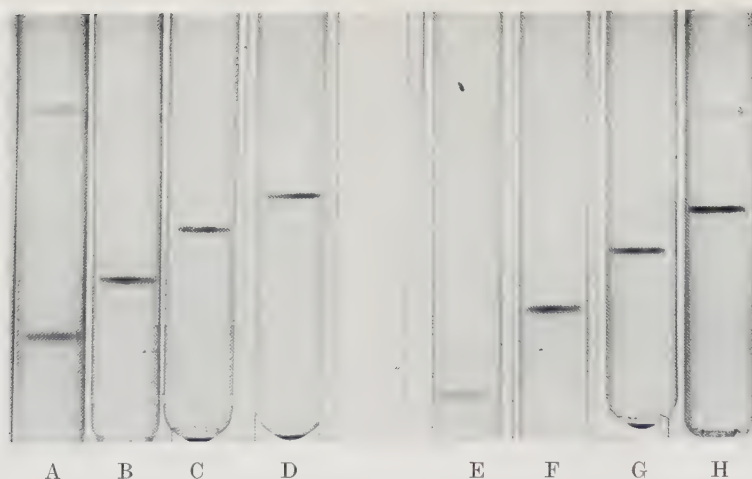


Fig.5. Polyacrylamide gel electrophoresis of E_I and E_{II} . Each form of the leucyl-tRNA synthetase (50 μ g) was loaded onto the top of the gel columns and electrophoresis was carried out as described in Methods. A, B, C, D: migration of E_I in 4.5, 6, 7.5, 9% acrylamide gels, respectively. E, F, G, H: migration of E_{II} in 4.5, 6, 7.5, 9% acrylamide gels, respectively



Fig.6. Comparison between E_I and E_{II} by gel electrophoresis. Standard gels (7.5% acrylamide) were used and the samples were analyzed as indicated in Methods. A: E_I (50 μ g); B: E_I (20 μ g) and E_{II} (40 μ g); C: E_{II} (50 μ g)

with respect to the ATP-PP_i exchange activity. In the same conditions, D_{II} yielded two peaks with the same catalytic and chromatographic properties as those obtained from D_I except that in this case HyB was about 25% of HyA.

When hydroxyapatite was replaced by DEAE-cellulose, similar results were obtained except that the positions of the peaks were reversed.

It appears from these results that the D_I and D_{II} fractions could be partially interconverted: the fraction inactive in the transfer reaction could be partially converted into the transfer-active fraction whereas the transfer-active fraction could be partially converted into the transfer-inactive fraction.

Chromatography of HyA on DEAE-cellulose gave two peaks, the first (E_I) unable to charge tRNA and the second (E_{II}) active in the transfer of leucine to tRNA. Here the first activity of the peak was about 2% of that of the second. When HyB was chromatographed in the same conditions, similar results were obtained except that the peak active in the charge reaction was 2% of the other peak with regards to the ATP-PP_i exchange activity. When hydroxyapatite was used in place of DEAE-cellulose, similar results were observed.

Thus the ability of one form of leucyl-tRNA synthetase to undergo partial transformation into the other form seems to decrease with increasing purity of the two forms of the enzyme, as if a factor favoring the interconversion between the two forms were lost during purification.

Evidence for the Existence of an Interconversion Factor

The same amount (1 exchange unit) of E_I and E_{II} was preincubated for various times with 0.2 mg protein of *E. coli* 105000 $\times$ g supernatant (previously heated for 2 hours at 55°, treatment which inactivated completely leucyl-RNA synthetase present in the supernatant) and the activity of each fraction for exchange and charge was tested. As shown in Fig.7, the ability of E_I to transfer leucine to tRNA increased with increasing time of preincubation with

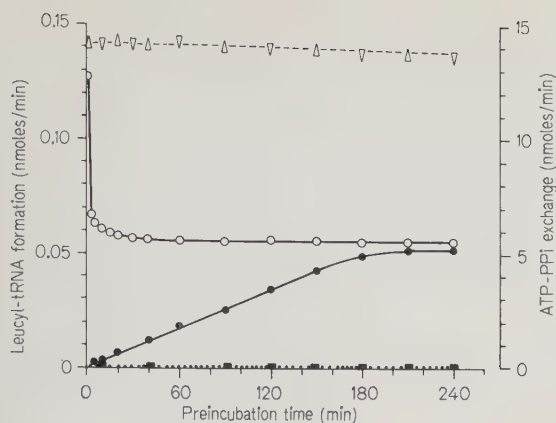


Fig. 7. Effect of $105000 \times g$ supernatant on the catalytic properties of E_I and E_{II} . Each enzyme (1 unit) was preincubated at 37° for various times with 0.2 mg of heated $105000 \times g$ supernatant and their activities were assayed as described in Methods. ●—●, E_I : leucyl-tRNA formation; ○—○, E_{II} : leucyl-tRNA formation; △—△, E_I : ATP-PP_i exchange; ▽—▽, E_{II} : ATP-PP_i exchange; ■·····■, S_{105} : leucyl-tRNA formation

the $105000 \times g$ supernatant whereas the charge activities of E_I and E_{II} reached the same state of apparent equilibrium but the kinetics were quite different. Under the same conditions, the exchange activity of each fraction was not significantly affected.

In order to further examine this interconversion phenomenon, E_I and E_{II} were separately incubated for 4 hours at 37° with heated $105000 \times g$ supernatant and were submitted to DEAE-cellulose chromatography in the same conditions as the controls incubated in the absence of supernatant. Fig. 8 indicates that incubation with $105000 \times g$ supernatant resulted in an interconversion between the two forms of the enzyme: E_I and E_{II} incubated with the supernatant were resolved into two peaks with respect to the ATP-PP_i exchange activity, only one of which was able to transfer leucine to the tRNA. Each of the controls yielded a single peak.

These results suggest that a factor stimulating the interconversion between the two forms of the synthetase is present in *E. coli* $105000 \times g$ supernatant, and is resistant to 2 hours incubation at 55° . Further experiments are being carried out in order to determine the nature of this factor and the conditions of the equilibrium between the two forms of leucyl-tRNA synthetase.

Properties of the Two Forms of the Leucyl-tRNA Synthetase

K_m values. Table 3 gives the K_m values for leucine and ATP; these results show that the K_m for ATP was the same for each enzyme form and that

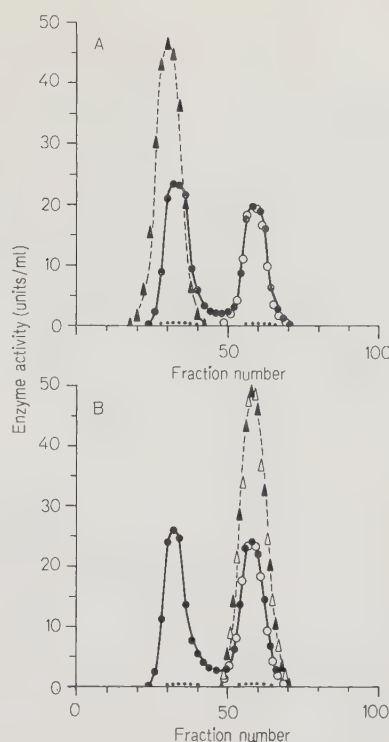


Fig. 8. DEAE-cellulose chromatography of E_I and E_{II} after incubation with $105000 \times g$ supernatant. Enzyme (1 mg) was incubated for 4 h at 37° with 50 mg of heated supernatant and loaded onto a 1×20 cm DEAE-cellulose column. A linear gradient (100:100 ml) from 0.05 to 0.3 M potassium phosphate pH 6.8 was applied to the column; 2 ml fractions were collected at a flow rate of 20 ml/h. As control, 1 mg of the enzyme was incubated for 4 h at 37° in the absence of supernatant and chromatographed as above. Another control was carried out with the same supernatant alone and chromatographed in the same conditions. (A) E_I ; (B) E_{II} . Enzyme plus supernatant: ●—●, ATP-PP_i exchange; ○—○, leucyl-tRNA formation. Enzyme alone: ▲—▲, ATP-PP_i exchange; △—△, leucyl-tRNA formation. Supernatant alone: ·····, ATP-PP_i exchange

Table 3. K_m values for leucine and ATP
The K_m values were determined by the ATP-PP_i exchange reaction in the conditions described in Methods

Enzyme	K_m (Leu)	K_m (ATP)
	μM	μM
E_I	120	720
E_{II}	87	710

the K_m for leucine was slightly higher with E_I than with E_{II} .

Formation of Enzyme-ATP and Enzyme-Leucyladenylate Complexes. Each form of the enzyme was able to form complexes to about the same extent with ATP and with leucyladenylate (Fig. 9). Only E_{II} was able to transfer leucine from the enzyme-leucyl-

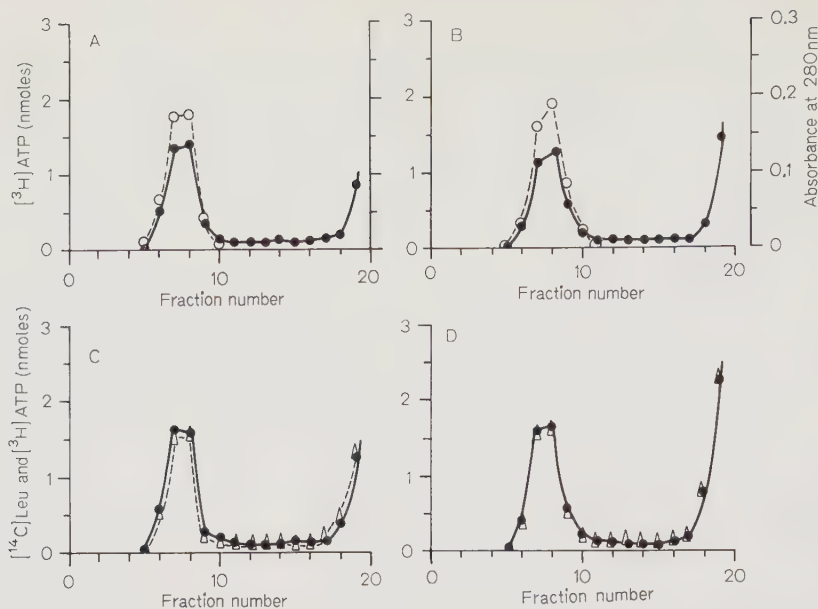


Fig. 9. Isolation of various complexes. The enzyme (0.5 mg) was incubated with 100 μ moles of [3 H]ATP and, when indicated, with 100 μ moles of [14 C]leucine, and then passed through a Sephadex G-75 column in the condition described in Methods. (A) E_I plus ATP; (B) E_{II} plus ATP; (C) E_I plus ATP plus leucine; (D) E_{II} plus ATP plus leucine. O---O, absorbance at 280 nm; ●---●, 3 H activity; △---△, 14 C activity

Table 4. Formation of complexes between leucyl-tRNA and either E_I or E_{II}

The enzyme (10 μ g) was incubated as described in Methods with the compounds indicated in the Table. The controls were incubated with or without 10 μ g of bovine serum albumin in place of the enzyme. The amounts of tRNAs were: crude [14 C]Leu-tRNA (0.4 mg—37 000 counts/min); crude tRNA (0.4 mg); [14 C]Leu-tRNA $^{Leu}_I$ (0.04 mg—31 000 counts/min); tRNA $^{Leu}_I$ (0.04 mg); [14 C]Leu-tRNA $^{Leu}_{II}$ (0.04 mg—34 000 counts/min); tRNA $^{Leu}_{II}$ (0.04 mg)

tRNA	Amount of complex formed with	
	E_I	E_{II}
	counts/min	
Crude [14 C]Leu-tRNA	6941	7482
Crude [14 C]Leu-tRNA + crude tRNA	3225	3640
[14 C]Leu-tRNA $^{Leu}_I$	6411	6917
[14 C]Leu-tRNA $^{Leu}_I$ + tRNA $^{Leu}_I$	3057	3231
[14 C]Leu-tRNA $^{Leu}_{II}$	6186	6783
[14 C]Leu-tRNA $^{Leu}_{II}$ + tRNA $^{Leu}_{II}$	2944	3304
Crude [14 C]Leu-tRNA + serum albumin without enzyme		147
Crude [14 C]Leu-tRNA alone		164

adenylate complex to either tRNA $^{Leu}_I$ or tRNA $^{Leu}_{II}$. This is an agreement with the conclusions of Bennet [2] that the same enzyme acylates these two *E. coli* tRNA Leu s.

Formation of Enzyme-tRNA Complexes. Although E_I did not charge tRNA, the incubation of E_I as of E_{II} with either *E. coli* [14 C]leucyl-tRNA $^{Leu}_I$ or [14 C]-

Table 5. Specificity of E_I for various amino acids
The ATP-PP $_i$ exchange reaction catalyzed by E_I in the presence of different amino acids was carried out as described in Methods

Amino acid	V_{max}	K_m
	nmoles/min	μ M
Leucine	10.7	120
Isoleucine	9.4	130
Valine	5.1	150
Methionine	2.3	810

leucyl-tRNA $^{Leu}_{II}$ led to the formation of a leucyl-tRNA... enzyme complex that could be isolated on nitrocellulose filters according to the method of Yarus and Berg [14], as shown in Table 4. The amount of the [14 C]leucyl-tRNA bound to the enzyme decreased by about 50% when uncharged tRNA $^{Leu}_I$ or tRNA $^{Leu}_{II}$ were added to the incubation mixture in the same amount as the corresponding 14 C-charged tRNAs, indicating that charged or uncharged tRNA-formed a complex to about the same extent with either form of the enzyme. The same result was obtained when crude [14 C]leucyl-tRNA and crude uncharged tRNA were used, indicating the specificity of E_I and E_{II} for tRNA Leu .

Specificity of E_I and E_{II} . As regards to the specificity of the two forms of leucyl-tRNA synthetase for amino acids, it was observed that E_{II} was highly

specific for leucine whereas E_I catalyzed the ATP-PP_i exchange reaction not only with leucine but also with isoleucine, valine and to a lesser extent with methionine. The K_m values of E_I for these amino acids are indicated in Table 5. E_I could not transfer any of these amino acids to the *E. coli* total tRNA. These results show that E_I has partially lost its specificity towards amino acids as compared to E_{II} . This loss of specificity is possibly due to the same conformational changes of the enzyme structure as that which are responsible for the loss of charge activity since, after conversion of E_I to E_{II} , it was observed that the specificity towards leucine and the charge activity are both recovered.

DISCUSSION

During the purification of leucyl-tRNA synthetase of *E. coli* B, two interconvertible forms of this enzyme were separated and purified to apparent homogeneity. Their molecular weights as determined by Sephadex filtration and gel electrophoresis were identical (about 104000) to the limits of experiments errors. One of these forms (E_I) was only able to catalyze the ATP-PP_i exchange reaction and was unable to acylate the tRNA, whereas the other form (E_{II}) catalyzed both reactions. As shown by their chromatographic and gel electrophoretic behaviour, E_I appeared less negatively charged than E_{II} .

The interconversion of the two forms of leucyl-tRNA synthetase appeared to be dependent on a factor present in the *E. coli* 105000 $\times g$ supernatant; after the incubation of E_I with the 105000 $\times g$ supernatant, a fraction of the enzyme became able to charge tRNA with leucine, whereas, after the incubation of E_{II} in the same conditions, a fraction of the enzyme was no longer able to charge tRNA. Starting with identical amounts of E_I and E_{II} as regards the leucine-dependent ATP-PP_i exchange activity, pre-incubation with the 105000 $\times g$ supernatant lead to the same level of charge activity, suggesting that an apparent equilibrium between the two forms of enzyme was reached. Also, when each form of the enzyme was preincubated with the 105000 $\times g$ supernatant in the same conditions, and then chromatographed on DEAE-cellulose, the elution profiles were identical and each form was resolved into two peaks, one of which was unable to transfer leucine to tRNA, whereas the other was able to catalyze this reaction.

The purification of the factor involved in the interconversion as well as the study of the kinetics of this reaction are investigated presently.

A partial lack of specificity of E_I for leucine was also observed since E_I catalyzed the ATP-PP_i exchange reaction not only with leucine but also with isoleucine, valine and methionine, whilst E_{II} catalyzed this reaction only with leucine. The recovery of the specificity was observed after incubation of E_I with

the 105000 $\times g$ supernatant, and it was concomitant with the transformation of E_I into E_{II} .

Either form of leucyl-tRNA synthetase was able to form complexes with ATP, leucyladenylate and with charged or uncharged tRNA^{Leu}_I and tRNA^{Leu}_{II} even though E_I was unable to charge tRNA with leucine.

It is possible that the differences between the two forms results from the arrangement of subunits. Indeed, our preliminary experiments suggest the existence of subunits in leucyl-tRNA synthetase. It should be noted that, for methionyl-tRNA synthetase the existence of subunits and of a dissociating factor was observed by Waller *et al.* [10,24]. Since both forms of the leucyl-tRNA synthetase are able to give complexes with their substrates, the orientation of subunits may be responsible for the relative position of tRNA and leucyladenylate on the molecule, thereby preventing or allowing the acylation of tRNA. The specificity and the apparent negative charge of the two forms of leucyl-tRNA synthetase may also be dependent on the arrangement of subunits.

It is not unlikely that the interconversion factor observed here induces conformational changes of the enzyme and that it is responsible for the relative orientation of the subunits. However, the mechanism of the conversion between E_I and E_{II} in the presence of the factor found in the 105000 $\times g$ supernatant as well as the physiological role of this factor remains to be elucidated.

We thank Mrs. Laura Litvak for her skilful and generous help in experiments with the interconversion factor. Part of this work has been supported by grants from the Délégation Générale à la Recherche Scientifique et Technique (Convention 6801348) and from the Commissariat à l'Energie Atomique (France).

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